Effects of electrical acupuncture stimulations on bone volume of rats' femur under different unloading conditions

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Abstruct

It has been reported that a bone formation is activated and a bone volume is increased by an electrical acupuncture stimulation after a tail-suspension. On the other hand, we recognized that a bone resorption was inhibited by the same stimulation during a hindlimb-immobilization. It could be assumed that conditions of mechanical load to hindlimb was different between the immobilization and the tail-suspension that could move hindlimb, and difference in the effect of the electrical acupuncture stimulation under those conditions haven't also been studied yet.

Then, in this study, effects of the electrical acupuncture stimulation on bone structural changes caused by the tail-suspension and the hindlimb-immobilization were investigated. Seven-week-old rats were used as materials. They were divided into five groups, that is, tail-suspended and immobilized group (Ts-Im), tail-suspended, immobilized and treated by an electrical acupuncture stimulated group (TI-EA), only tail-suspended group (Ts-Mo), tail-suspended and electrical acupuncture stimulated group (TM-EA) and control (CO). Femurs were excised from each group after 2-week-experiment, various specimen were analyzed morphologically.

Many bone resorption images were found in a periosteum side of a cortical bone and, blood vessel cavities and osteocytic lacunas expended, in Ts-Mo and Ts-Im. Those

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changes were inhibited by the electrical acupuncture stimulation. Decrease in the bone volume of Ts-Im was conspicuous, compared to Ts-Mo.

It was understood that the immobilization decreased obviously the bone volume, compared to the tail-suspension, and the electrical acupuncture stimulation contributed to maintain the bone volume.

Keywords: electrical acupuncture stimulation, mechanical loading, bone volume.

1. Introduction

Our country reaches a super aging society, and medical fiscal pressure becomes the social problem. The health problem of the elderly person is same, too. It is mentioned that a bone fractures by an osteoporosis cause a state of being bedridden, following the apoplexy.

The health maintenance of the bone is regarded as an important factor in planning extension of the healthy life expectancy[1]. Development advances to the therapeutic drug of the osteoporosis, and a bone formation accelerating-agent like a parathormone and a bone resorption inhibitor such as bisphsphnate or denosumab accelerant are used generally.[2]

Furthermore, it has been reported that the mechanical stress by the exercise practice is effective on a bone mass increase[3]. On the other hand, treatment promotion effects of an electrical acupuncture stimulations on soft tissue, like local bloodstream increase[4] and healing of rupture of Achilles' tendon[5], have been reported[6,7].

The effects of the electrical acupuncture stimulation on a maintenance or increase of a bone volume were reported by studies using the bone fracture model of the rat's tibia[8] and the tail-suspension model[9]. While a bone formation was promoted by the electrical acupuncture stimulation after the tail-suspension in the report of Lam and Qin[9], we reported that the bone resorption was inhibited by that stimulation during a hindlimb-immobilization[10].

However, there are few histological studies about the effects of the electrical acupuncture stimulation on the bone.

Purpose of this study was, using rat's femur, to compare and investigate the effects of the acupuncture electricity stimulation on the bone structures during the tail-suspension and the hindlimb-immobilization.

2. Materials and methods

2.1 Materials

Forty male rats (wistar strain, seven-week-old) were used as materials and were divided into two groups, that is, experimental group (Ex) and control group (CO) .

2.2 Conditions of mechanical unloading

Ex were, furthermore, divided into (1) tail-suspended and immobilized group (Ts-Im) , (2) tail-suspended, immobilized and electrical acupuncture stimulated group (TI-EA), (3) only tail-suspended group (Ts-Mo) , and (4) tail-suspended and electrical acupuncture stimulated group (TM-EA) . Each treatment was performed for two weeks in Ex, and rats of CO were fed ordinarily for the same period.

2.3 Acupuncture portion and conditions of electrical acupuncture stimulation

Following our previous study[10] , an acupuncture was inserted until a periosteum and continuous electrical stimulations (250μsec、50Hz、0.24mA) were given for 10 minutes /day, every day for two weeks, in TI-EA and TM-EA. (Fig 1)

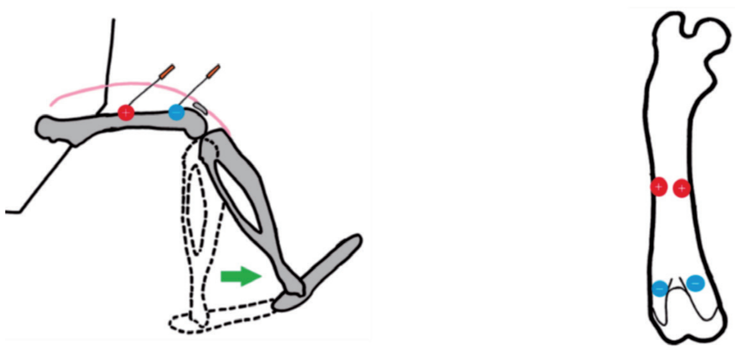

Fig.1. acupuncture stimulating portion (from ref.10)

Left: side face of hindlimb, Right: anterior face of femur

2.4 Sampling and fixation

Rats of each group were euthanized by CO_2 gas and their muscles, vessels and nerves were excised from femurs after confirming death. Right legs were used for bone strength tests and left ones were used for structural observation and bone morphometry.

Distance from major trochanter to lateral condyle was measured as a bone length of femur, and middle and distal 1/3 portions of femur were decided based on those data.

Three-point-bending tests of right femurs were performed without fixation immediately after excise. Left femurs were cut transversally at the middle and distal portions, and furthermore, these samples were trimmed by cut transversally or longitudinally. Former specimen was used for preparing of resin-embedded ground section and later specimen was used for preparing for paraffin-embedded sections and scanning electron microscopy. They were immersed in fixation fluids (4%paraform aldehyde or Karnovsky fluid) immediately after trimming. Those fluids were buffered by 0.1M cacodylate buffer (pH7.4).

2.5 Bone morphometric

Cross-sectioned ground sections of femur embedded in resin were stained by toluidine blue dye, by methods described below.

Photos of those sections were analyzed by image analyzing application (WinROOF V7.4) according to general methods[11-13].

Anterior-posterior (A-P) and medial-lateral (M-L) diameters and cross-sectional area of the cortical bone were measured using cross-sections of femur.

2.6 Conditions of three-point bending test

Parameters of three-point bending test were calculated from relation curve (L-M curve) showing relation between the mechanical loading and the bone modification[11-13]. Stiffness was one of the parameters and was calculated from an inclination of that curve. Strength was regarded as a maximal load (N) exceeding elastic limit. Deformation was assumed as bone modification at the maximal loading.

2.7 Relative values of parameters of three-point bending test (secondary parameters)

Secondary parameters were calculated based on results of the bone morphometry and strength tests. It was showed that Strength was proportional to the cross-sectional area and Deformation was in proportion to the diameter[14]. Therefore, Strength per unit area, that is, relative value of Strength was calculated by dividing Strength by the cross-sectional area. Relative value of Deformation was calculated by dividing Deformation by the diameter (A-P).

2.8 Histological preparation and observation of specimen

Specimen for observing tissue were used for preparations of paraffin sections and specimens of SEM. The specimens for the paraffin sections were immersed in 8% EDTA solution (pH7.4) or Mose's solution for two weeks and were decalcified.

Those specimens were rinsed, dehydrated at vacuum condition, cleared by methyle broate and benzene. And then, they were embedded in paraffin wax.

Four-micrometer-thickness paraffin sections were cut by a microtome and were stained by polychrome staining method. The specimens that were fixed by later fluid were dehydrated without decalcification, were cleared by acetone, were embedded in the resin and were polymerized by heating.

After trimming of that polymerized block, they were cut in sagittal and horizontal directions and were ground by stones and films for grinding. They were etched by 1N HCl, were rinsed and were stained by toluidine blue dye.

The specimen embedded in paraffin wax were sectioned and they were stained by TRAP (Tartrate resisted acid-phosphate) stain method.

Those decalcified and undecalcified specimen were observed by a light microscope.

2.9 Classification of bone matrix

Ohsako et al.[15] divided a bone matrix according to a density and an arrangement of matrix fibers based on observations by light microscope and transmission electron-microscope. It was shown, in this report, that the matrix fibers were loose at the area stained pale blue and were dense at area stained red by Masson-tricrome staining method, and in addition to those areas, which showed a lamellar structure also existed in the bone.

Moreover, it was known that the stainability of the bone matrix stained by toluidine blue dye was deferent between a calcified cartilage matrix and the bone matrix. Former matrix caused metachromasia[16,17] and were stained violet because that matrix contained acid mucopolysaccharides[18] .

Therefore, in this study, the area consisted with loose and irregular-arrangement matrix fibers was assumed type A, the area showed the lamellar structure was regarded as type C, the area indicated intermediate structure among type A and C was assumed type B[15] .

2.10 Statistics

Averages and the standard deviations of each parameter of the bone morphometry were obtained and differences of the average between the groups were calculated.
Significant differences were calculated by Mann-Whitney U test using the SPSS software.
Significance of difference of averages was considered below 5 % of standards.

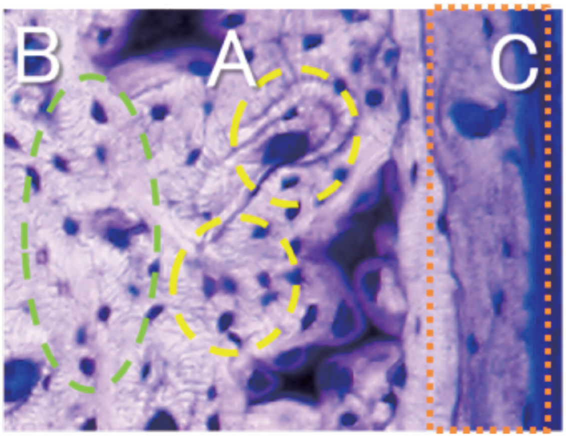

Fig.2. Typical structures of the bone matrix of cortical bone

Bone of type A consists of loose and irregular arranged fibers. Bone of type C shows lamellar structures. Bone of type B is intermediate type of type A and C.

3. Observations

3.1 Macroscopic observations

The femur and muscles attaching to it were stained by toluidine blue dye after fixation. The thigh extensor mm., the flexor mm. and the adductor mm. surrounded the neighborhood of the bone. The tendon-fiber of quadriceps femoris m. that is situated on the thigh front arranged in the axial direction of the length of the bone and attached to the bone loosely to the periosteum surface from near parallel direction.

The tendon-fiber of the adductor mm. which attached to the posterior and lateral face of the femur was arranged vertically to the bone surface and the periosteum of such tendon attachment site was thick. The muscles were exfoliated from the periosteum at the thin part of it, but an exfoliation between the muscle and the periosteum was difficult at the part with thick periosteum.

3.2 Bone morphometry

3.2.1 Cortical bone

As for anterior-posterior and medial-lateral diameter at middle and distal portions of femur, CO was at the same level as TMEA and TIEA. On the other hand, Ts-Im, Ts-Mo which wasn't treated by the acupuncture electricity stimulation showed the significant low values, compared to the other group. (Table 1.) A cross-sectional area also decreased significantly by the non-weighting compared to CO but TMEA and TIEA that were treated by the acupuncture stimulation maintained this parameter. (Fig 4., Fig 5.)

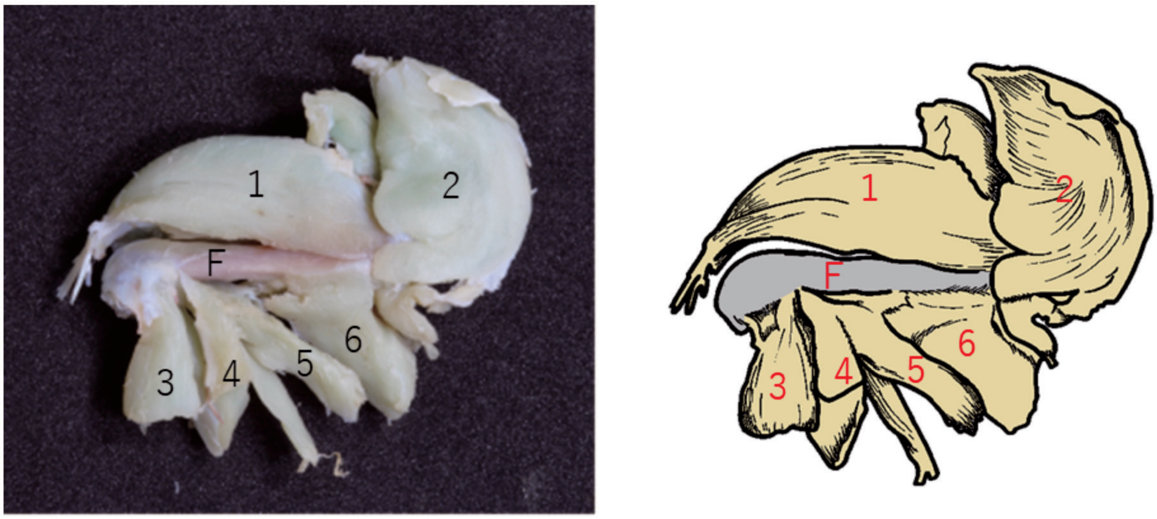

Fig.3. Muscles attaching to femur

Left: Photo of lateral side face, Right: Trace of left photo
F: femur, 1: femoral quadriceps m. 2: profound gluteal m. 3. gastrocnemius m.
4. semitndinous m. 5. semimembranous m. 6. great adductor m.

Table 1. Morphometric data of middle and distal portions in each group

Middle (n=8)	CO	Ts-Mo	TMEA	Ts-Im	TIEA
A-P dia. (mm)	3.21±0.07	3.02±0.10	3.13±0.11	2.94±0.08	2.99±0.13
M-L dia. (mm)	4.43±0.13	3.96±0.12	4.13±0.17	4.01±0.08	4.09±0.14
Area (mm^2)	4.86±0.25	3.75±0.26	4.26±0.25	3.40±0.12	4.07±0.20

Distal (n=8)	CO	Ts-Mo	TMEA	Ts-Im	TIEA
A-P dia. (mm)	3.44±0.05	3.35±0.22	3.41±0.15	3.07±0.10	2.99±0.18
M-L dia. (mm)	4.73±0.11	4.58±0.29	4.62±0.23	4.54±0.17	4.34±0.19
Area (mm^2)	4.60±0.17	3.45±0.18	3.58±0.33	3.03±0.15	3.48±0.30

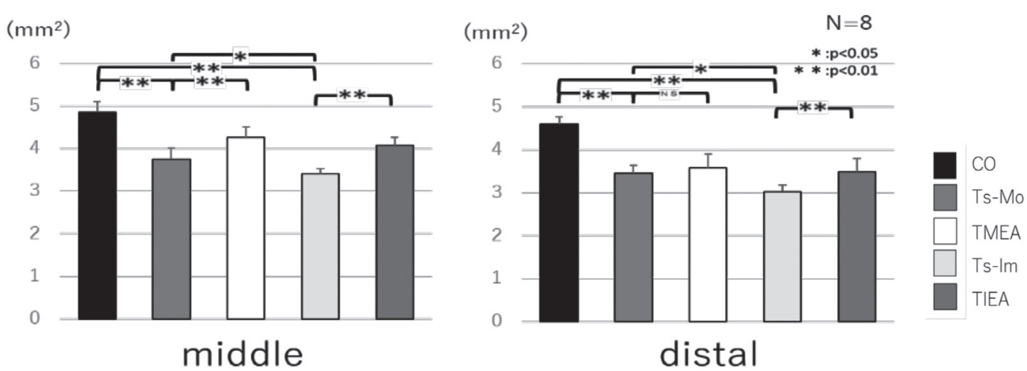

Fig.4. Bone morphometric data in each group

3.2.2. Osteocytic lacunas

Sizes of osteocytic lacunas were larger at the distal portion than at middle portion in every groups. Ts-Mo and Ts-Im showed the significant higher values than CO and TIEA was as the same CO. (Table 2.)

Table 2. Sizes of the osteocytic lacunas in each group

(n=6)	CO	Ts-Mo	TMEA	Ts-IM	TIEA
middle	25.33 ± 0.98	34.6 ± 2.33	32.45 ± 1.72	34.61 ± 0.92	25.43 ± 1.40
distal	28.62 ± 2.09	37.61 ± 3.95	37.9 ± 1.38	46.69 ± 1.77	32.25 ± 1.18

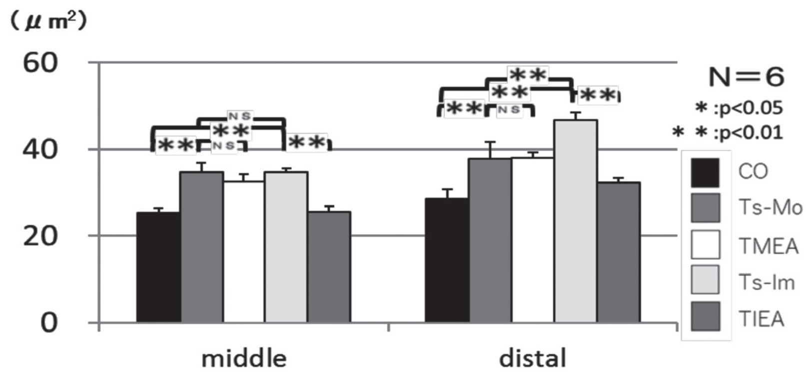

Fig.5. Comparison of osteocytic lacunas in each group

3.3. Histology

3.3.1 Resin-embedded undecalcified ground section

When seeing the cross section of the T cortical-bone in low-magnified images (× 12.5), Thickness of the cortical bone was thin at the posterior-lateral face of the middle portion in Ts-Mo and especially Ts-Im. To the contrary, thickness of that was maintained in groups that were treated by the acupuncture electricity stimulation.

Areas stained darkly by toluidine blue dye were observed at the anterior face of the distal portion in Ex, except for TIEA. At those areas, the bone matrix was stained blue, differed from the calcified cartilage matrix stained violet or an osteoid stained pale blue. (Fig. 6.)

The lamellar structures were recognized in CO and TIEA when observing their magnified images. However, those structures weren't found and pectinate or reticular structures appeared at endosteum surface in the groups except for those groups. There were no calcified cartilage matrixes in this area of every groups.

There were developed lamellar bone in the posterior –latelar face at of middle portion

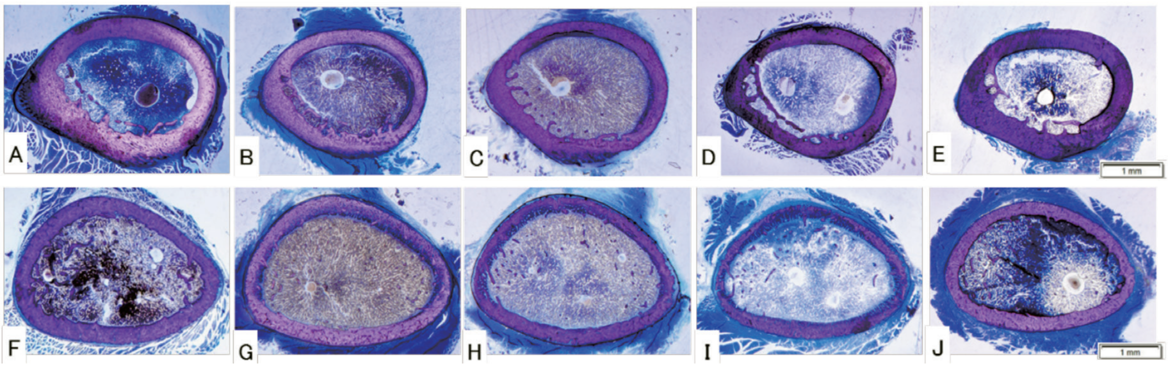

Fig.6. Morphology of femur at the middle and distal portions in each group

Thickness of the cortical bone was thin at the posterior-lateral face of the middle portion and at the anterior face in Ts-Mo and especially Ts-Im. On the other hand, thickness of that was maintained in groups that were treated by the acupuncture electricity stimulation.

A-E : middle potion F-J : distal potion

A, F : CO B, G : Ts-Mo C, H : TMEA D, I : Ts-Im E, J : TIEA

the femur. Furthermore, the blood vessel cavities in Ts-Mo and Ts-Im expanded, compared to CO. (Fig 7.)

There were no differences in distribtution of the bone types in the cortical bone at the middle portion. However, the bone matrix of typeA was resorbed selectively in tail-suspended groups. The bone matrix stained by toluidinble dye, described above,

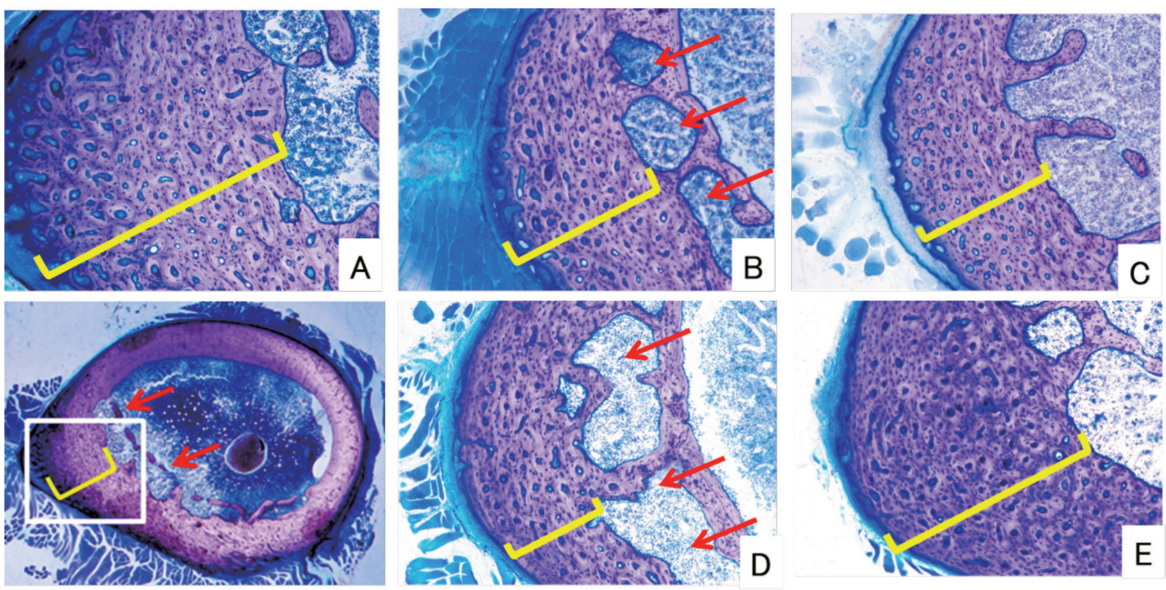

Fig.7. Comparison of structures of the cortical bone magnified in each group

Extended blood vessel cavities were found at endosteum side of the cortical bone in Ts-Mo and Ts-Im.

yellow frame: thickness of cortical bone red arrow: Vessel cavity

A : CO B : Ts-Mo C : TMEA D : Ts-Im E : TIEA appeared at the distal portion in Ts-Mo, Ts-Im and TMEA. (Fig 8.)

Differences were found in size and shape of the osteocytic lacunae depended on the portions of the cortical bone. Those lacunas were the smallest at the deep layer of the

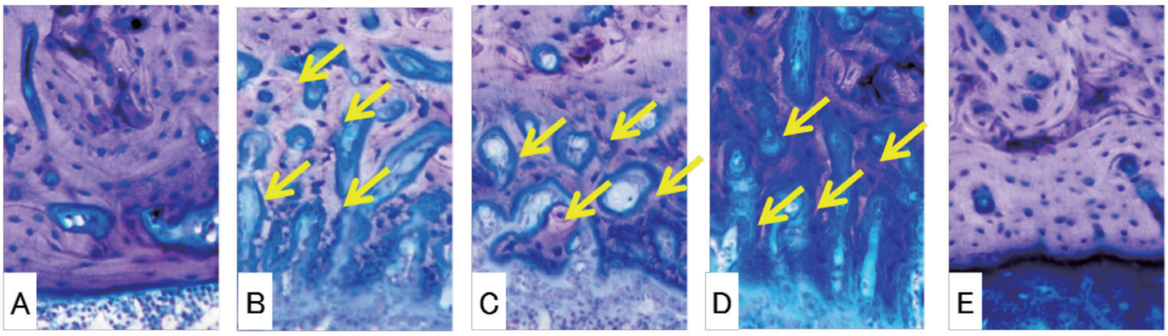

Fig.8. Distribution of various types of the bone matrix in the cortical bone

A : CO B : Ts-Mo C : TMEA D : Ts-Im E : TIEA

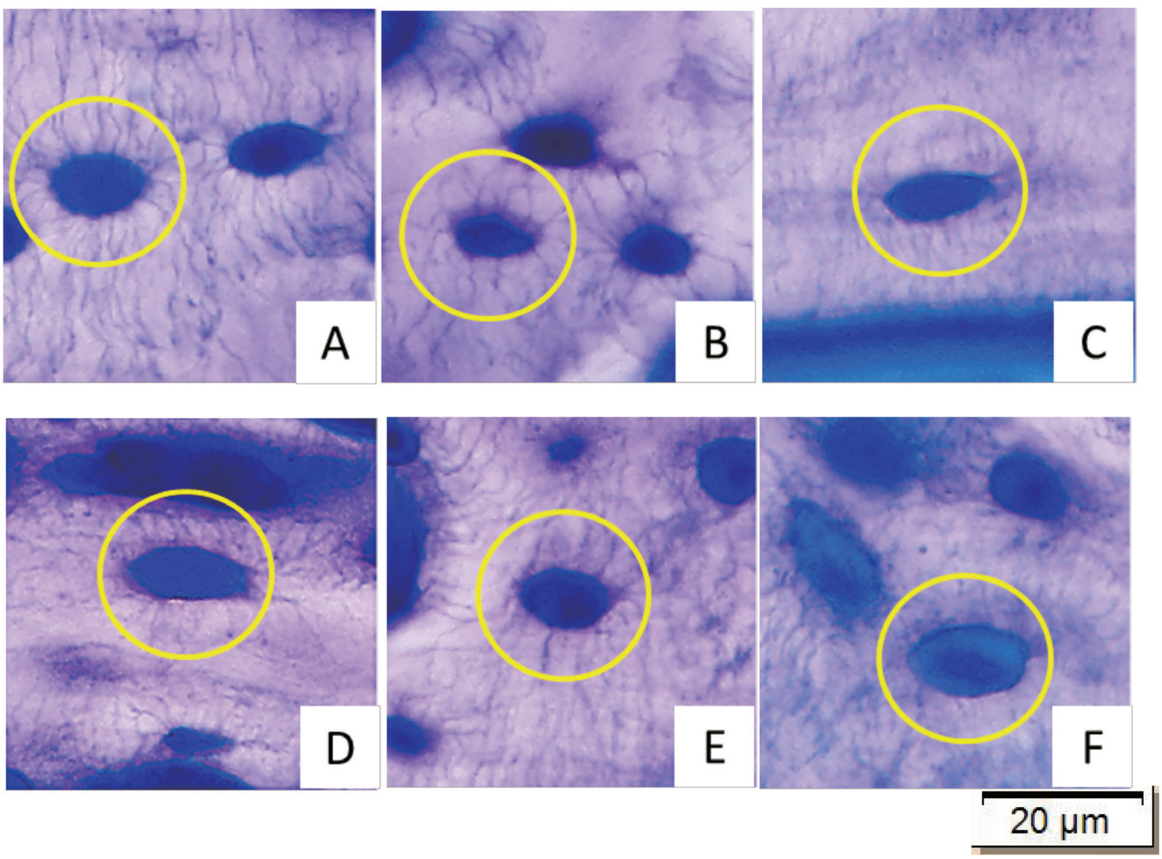

Fig.9. Shapes of osteocytic lacunae in CO

Yellow circles indicate the osteocytic lacunas.

Shape and size of the osteocyte were different between portions in the cortical bone.

A, B, C : middle potion D, E, F : distal potion

A, D : Periosteum potion B, E : Deep potion C, F : Endosteum potion cortical bone, when dividing the bone into periosteum, deep and endosteum layers from the periosteum side.

Distance between osteocytic lacunas was narrow and they existed dense in type A that existed at the periosteum side of the cortical bone. The osteocyte lacunas existing in type B at the periosteum side of middle portion showed a spindle –shape and it were same as those at the periosteum side of distal portion. They existing in type C at the endosteum side of the middle portion showed slight long spindle-shape. (Fig.9)

3.4. paraffin embedded decalcified sections

Osteoclasts and large and small Howship's groove existed at the anterior face of femur when observing sections stained by TRAP staining method. Many TRAP- positive-cells were recognized clearly at the distal portion, compared to at the middle portion.

Furthermore, when comparing between inter-groups, there were many TRAP- positive-cells in Ts-Mo and Ts-Im but were little in TMEA and TIEA especially that were treated by the electrical acupuncture stimulation. (Fig 10.)

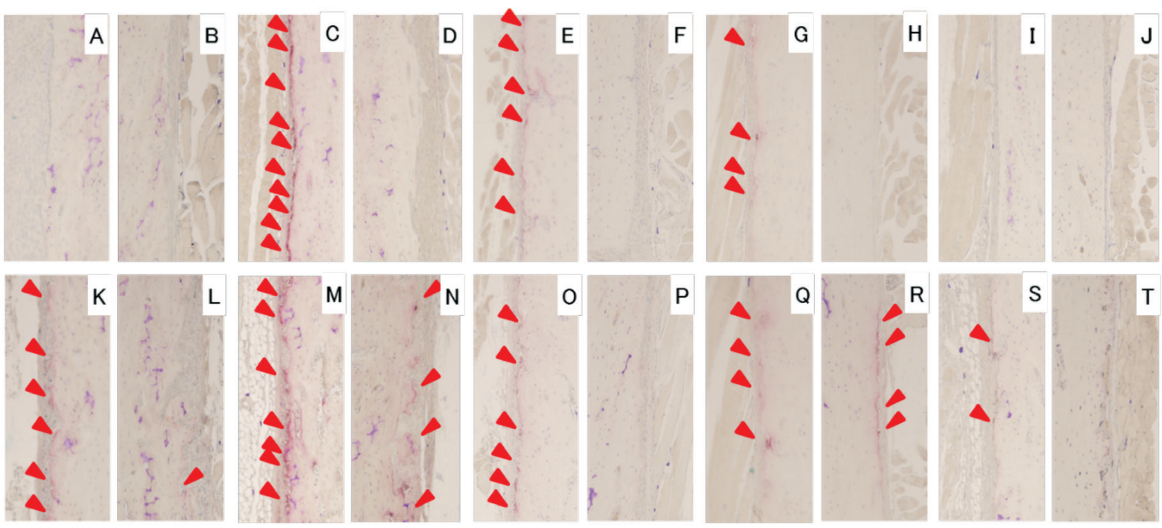

Fig.10. Bone resorption images at the anterior and posterior face in the periosteum side.

Many TRAP-reaction positive cells (arrow heads) were found in Ts-Mo and Ts-Im but they were little in TMEA and TIEA.

A-J：middle potion　K-T：distal potion

A, C, E, G, I, K, M, O, Q, S：anterior potion　B, D, F, H, J, L, N, P, R, T：posterior potion

A, B, K, L：CO　C, D, M, N：Ts-Mo　E, F, O, P：TMEA　G, H, Q, R：Ts-Im　I, J, S, T：TIEA

3.5. Bone strength test

Ex showed lower values of Stiffness than CO. On the other hand, groups that were treated by the electrical acupuncture stimulation indicated same level of Deformation as CO. Values of Strength in Ex were lower than CO. (Table 3., Fig 11.)

Table 3. Results of bone strength test in each group

3PB (n=8)	CO	Ts-Mo	TMEA	Ts-Im	TIEA
STIFFNESS (N/mm)	225.4±16.9	131.7±31.5	144.5±21.0	156.9±30.7	182.1±35.0
DEFORMATION (mm)	1.21±0.04	1.10±0.08	1.18±0.09	1.03±0.09	1.10±0.16
STRENGTH (N)	95.0±5.9	68.4±5.7	73.0±11.4	66.0±8.2	76.8±4.5

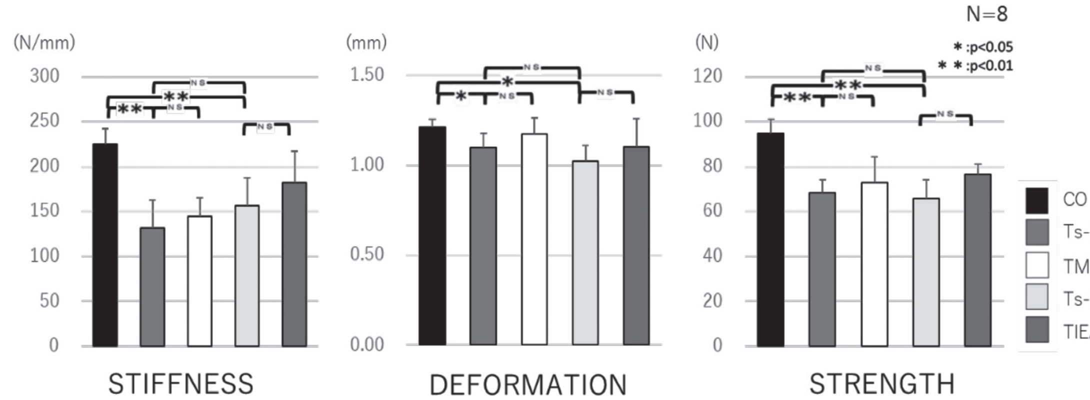

Fig.11. Comparison of data of bone strength test in each group

3.6. Relative values of parameters of three-point bending test (secondary parameters)

Relative values (Def./Dia.) were almost same in every groups, but Strength per area of CO was higher than Ex. Furthermore, values of length ratio anterior-posterior to medial-lateral (AP/ML) were lower in Ts-IM and TIME. (Table 4)

Table 4. Secondary parameters of bone strength tests

CO	Ts-Mo	TMEA	Ts-Im	TIEA	TIEA
str./area	19.6	18.2	17.1	19.4	18.8
def./dia.	0.38	0.36	0.38	0.35	0.37
mid (AP/ML)	72%	76%	76%	73%	73%
dis (AP/ML)	73%	73%	74%	68%	69%

4. Discussion

It has been reported that a bone volume and surrounding soft tissues declined by decrease in mechanical stress such as hindlimb immobilization[19] and tail-suspension[20].

It was also showed that a suitable exercise enhanced a bone formation ability but higher intense exercise[21] or prolonged exercise[22] inhibited it.

On the other hand, Ikemune et al[7]. reported that the electrical acupuncture stimulation maintained a muscle wet weight by inhibiting expression of myostatin that controlled increase in muscle. In this study, effects of the electrical acupuncture stimulation during tail-suspension and hindlimb-immobilization were investigated by observing the structural changes in the bone tissue, and were discussed blow based on the results.

The femur was surrounded by femoral extensor, flexor and adductor muscle group.

Tendon fibers of femoral quadriceps m. arranged from near parallel direction of long axis of the femur and attached to an anterior face of that bone loosely. The tendon fibers of the adductor muscle group attached to the femur from near perpendicular direction of the long axis of that bone and the periosteum was thick at the attaching portion. The muscle was peeled easily from the surface of periosteum because connection between both tissues were loose at the area that the periosteum was thin. To the contrary, it was difficult to peel the muscle from the periosteum because the connection between both tissues was strong.

It was reported that decrease in the mechanical stress affected condition of the muscle-attaching[23], and this was important to understand the morphological data. The length in anterior-posterior direction of the cross-sectioned face at the middle and distal portion in CO was same as TMEA and TIEA that were treated by the electrical acupuncture stimulation but Ts-Im and Ts-Mo that didn't receive that treatment showed significant low values of that.

Large effect on the length of medial-lateral direction of the cross-sectioned face was recognized, compared to the length of anterior-posterior direction of that. It was thought that this was derived from tractive force of the muscles that attached to a trochanter tertious because osteoblasts were activated by that force functioned the bone[24]. Furthermore, it was supposed that difference in the structure of the attaching portion between TMEA and TIEA reflected whether spontaneous exercise was possible or not.

Groups that didn't received the treatment of the electrical acupuncture stimulation showed lower values of the cross-sectional area than CO, but those values were maintained in the group that received that treatment. It was thought that decrease in the mechanical loading and immobilization caused enhancing of the bone resorption ability of osteoblasts and suppression of bone formation by osteoblasts and resulted in loss of the bone volume. Oppositely, it was speculated that the bone volume was maintained due to the tractive force from the muscles caused by the electrical acupuncture stimulation.

The areas of the oseocytic lacunas were larger at the distal portion than at the middle portion. An endochondral ossification was progressed at the distal epiphysis in the case of femur at the growing period and the bone formation was progressed from distal growth plate toward middle portion. It was supposed that difference in size of the oseocytic lacunas between both portions related to process of the bone formation. The lacunas of Ts-Mo and Ts-IM that weren't stimulated by the electrical acupuncture stimulation in the anterior were larger than CO and those of TIEA were at the same level as CO.

It was showed that mineral substances eluted from walls of the ostecytic lacunae and canaliculi by administration of a parathyroid hormone (PTH), and their lumen expanded[25,26]. Same changes in the osteocytic lacunas were recognized in the group that were treated by the tail-suspension and hindlimb-immobilization and those changes were inhibited by the electrical acupuncture stimulation, in this study. Therefore, it was assumed that the electrical acupuncture stimulation could have an effect on suppressing of osteolysis under the condition of decrease in the mechanical stress.

Large bone marrow cavities were found at the endosteum side of the cortical bone and the cortical bone was thin in the groups that only hindlimb-immobilization was treated. It was thought that the bone was resorbed actively in the endosteum side, and at the same time, the bone formation was inhibited by the electrical acupuncture stimulation in these groups.

The cortical bone was thick in the posterior face in CO, TMEA and TIEA, and on the other hand, was thick at the muscle-attaching portion in CO, Ts-Mo and TMEA. Then, it was supposed that the tractive force from the muscle affected to thickness of the cortical bone.

Many TRAP-reaction-positive cells were observed in Ts-Mo and s-Im but little those cells were found in TMEA and TIEA that were treated by the electrical acupuncture stimulation. It was thought that this meant inhibition of the bone resorption activity by the osteoclasts due to that treatment.

It was known that Stiffness related to calcifying degree and density of collagen fibers , and Stiffness increased but Deformation declined by ascending of the calcifying degree. This related to growth of mineral crystals attached to bone matrix fibers, and the crystals grew after the bone formation, too, and this was one of factors of Stiffness increase. It was thought that increase in crosslinking (AGEs) of the collagen fibers with aging raised Stiffness and resulted in fragile bone[27] .

Stiffness in Ex was lower than that in CO but Deformation in Ex was same level as CO. Ex showed lower values of Strength, compared to CO, but it maintained to the extent in the groups that were treated by the electrical acupuncture stimulation.

The bone is differed from homogeneous structures like a metal and a resin. Then, relative values of Strength and Deformation were calculated due to dividing those parameters by the cross-sectional area of the cortical bone[14). Those relative values were different between each group in this study, and it seemed be that this reflected differences in the bone volume and the structures.

It was showed that the bone having typical lamellar structure was higher degree of the histological differentiation and long time was necessary for forming this bone when considering about rate of the bone formation and tissue differentiation from the point of view of density and arrangement of the bone matrix fibers[15).

The influences of different conditions of mechanical loading were observed at the posterior portion, that is, undifferentiated immature portion. It was supposed that rate of metabolic-turnover the influences of the immobilization because obvious were found at the portion that not only the active bone formation but also were performed at the same time, that is, the portion of high-turnover.

5. Conclusion

It was understood that the immobilization decreased obviously the bone volume, compared to the tail-suspension, and the electrical acupuncture stimulation contributed to maintain the bone volume.

Summary of this study was reported at 121th Japanese Association of Anatomics in Fukushima.

6. Committee of Animal Experiment and Ethics

This study was approved by Committee of Animal Experiment and Ethics for the research, Graduate School of Welfare Society design, Toyo University.

7. Acknowledements

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異なる非加重条件によるラット大腿骨の骨量減少に対する鍼通電刺激の影響

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要　旨

通電刺激により骨形成が促進し骨量が維持されることが報告されている。一方、我々は後肢不動中に鍼通電刺激を行い、その結果、骨形成の促進よりも骨吸収が抑制されることにより骨量の維持を認めている。ベッドレストを想定し、非加重下において自動運動できる状態と不動状態とでは、下肢骨への加重条件が異なることが考えられ、それらに対して鍼通電刺激による骨量維持の程度を組織学的に比較、検討した報告はない。

本研究は、ラット大腿骨を用いて尾部懸垂中に後肢不動化または加重低減中に大腿部への鍼通電刺激を施し、それらの骨構造の変化を観察することを目的とした。

７週齢のウィスター系雄性ラットを８匹ずつ以下に述べる５群に分け２週間実験を行った。尾部懸垂-不動群（Ts-Im）、Ts-Im-鍼通電刺激群（TI-EA）、尾部懸垂-可動群（Ts-Mo）、Ts-Mo-鍼通電刺激群（TM-EA）、それらに対する対照群（CO）とした。実験終了後、速やかに大腿骨を摘出し、骨幹中央部の強度試験と組織観察用標本の作製および観察を行った。

Ts-Im・Ts-Moでは、皮質骨の骨膜側の吸収像が多く認められ、骨内膜側の血管腔や皮質骨内の骨小腔の増大化がみられた。TI-EA、TM-EAではそのような骨構造の変化が抑制され、強度の維持に影響を及ぼしていることが示唆された。

Ts-Moに比べ、Ts-Imの減少量は大きく、鍼通電刺激を施すことで同程度に骨量維持された。さらに組織学的に骨内膜面の骨吸収が抑制されることが理解された。

キーワード：鍼通電刺激、加重低減、骨量減少抑制